October 1947
Revised March 1948
Revised February 1950
Reissued with supplement October 1950

AIC-168

ANNOTATED BIBLIOGRAPHY OF SUBTILIN: Assay, Microbiological Production, Purification and Chemistry, Biological Activity, and Related Compounds

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ANNOTATED BIBLIOGRAPHY OF SUBTILIN

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The genus Bacillus has a long record of observed antibiotic activity. This bibliography covers reports of the antibiotic activity produced by a particular strain of Bacillus subtilis, first studied from this aspect in the Western Regional Research Laboratory, and of the antibacterial agent, subtilin, concentrated from cultures of this organism. This strain of B. subtilis was originally obtained from N. R. Smith of the U. S. Department of Agriculture, Bureau of Plant Industry, Soils, and Agricultural Engineering, under the number 231. It is now deposited in the American Type Culture Collection as number 6633 and in the culture collection of the Northern Regional Research Laboratory as B-543.

Those articles originating from the Western Regional Research Laboratory are marked "WRRL" following the authors' names.

Assay

Most of the work on subtilin has been done with concentrates. The most potent preparations made at the Western Regional Research Laboratory have appeared nearly homogeneous by electrophoresis, by fractional dialysis, and by fractional precipitation with sodium chloride (see Fevold et al, Page 6). On the other hand, investigations at the research laboratories of Merck & Company, Rahway, New Jersey, by countercurrent distribution, show that our most potent preparations are probably about 90 percent pure on the basis of the increased bacteriostatic potency of the principal fraction. The arbitrary subtilin standard, lot L1263, referred to in many of the papers from the WRRL, accordingly is 38.5 percent as potent as pure subtilin by turbidimetric assay with Micrococcus conglomeratus (see next below). Editorial insertions regarding potency of subtilin preparations used in various studies are indicated by brackets. Sensitive organisms for the cup-plate assay of subtilin and subtilin esters are mentioned by Wilson et al (Page 12).

Lewis, J. C., Humphreys, E. M., Thompson, P. A., Dimick, K. P., Benedict, R. G., Langlykke, A. F., and Lightbody, H. D., WRRL (authors Benedict and Langlykke, WRRL). The microbiological assay of subtilin. Arch. Biochem. 14(3):437-450, 1947. Subtilin was assayed by a turbidimetric method which depends on growth inhibition of Micrococcus conglomeratus (MY), Streptococcus faecalis (ATCC 7080), or Staphylococcus aureus (H). The cultures are incubated for 4 or 5 hours at 37° C. under nonsterile conditions. The antibiotic activity of crude culture extracts of B. subtilis (ATCC 6633) and of partially purified lots of subtilin could not be accounted for entirely in terms of a single active substance. Sixty to 70 percent ethanol was optimal for extraction of subtilin

from crude cultures. The extraction was essentially complete in 15 minutes. Crude cultures lost substantial amounts of activity on steaming for 1 to 2 hours; the rate of destruction was faster at pH 6.4 to 7.1 than at 2.5 (Reprints exhausted.).

Housewright, R. D., Henry, R. J. and Berkman, S., Camp Detrick, Md. A microbiological method for the assay of subtilin. Jour. Bact. 55(4): 545-550, 1948. A diffusion plate assay which utilizes filter paper discs and Bacillus cereus 247 is described. In the range of 15 to 32 mm., zone diameter is linearly related to log subtilin concentration. Although precision is favored by high slope (doubling the concentration increased the zone diameter by 5.3 mm.), the method has low sensitivity. A 20 mm. zone required 200 ppm. of subtilin lot 118-T [45 percent as potent as pure subtilin by turbidimetric assay]. Increased zone diameter is favored by low pH in the range of 5.9 to 7.2, and by high NaCl concentration in the range of 0 to 2 percent.

Microbiological Production

Rumfeld, H., and Feustel, I. C., WRRL. Utilization of asparagus juice in microbiological culture media. Proc Soc. Expt. Biol. and Med. 54(2): 232-235, 1943. B. Subtilis (ATCC 6633) cultures grown for 24 hours at 35° C. on asparagus butt juice medium were adjusted to pH 2.5 and sterilized for 10 minutes at 10 lbs. of steam pressure. These preparations possessed marked antibiotic activity against Staphylococcus aureus, Micrococcus conglomeratus, Lactobacillus casei, and the plant pathogen, Phytomonas michiganensis. Less activity was found against the Gram-negative plant pathogens Phytomonas juglandis and Erwinia amylovora. (Reprints exhausted.)

Jansen, E. F., and Hirschmann, D. J., WRRL. Subtilin--An antibacterial product of Bacillus subtilis. Culturing conditions and properties. Arch. Biochem. 4(3):297-309, 1944. B. subtilis (ATCC 6633) was grown in surface cultures on media containing sucrose, mineral salts, and several organic nitrogen sources including asparagine, glutamic acid, aspartic acid, casein, and tryptone. The addition of manganese (1ppm.) proved essential for high antibiotic activity. Wide variations were noted in semi-quantitative serial dilution tests of anti-bacterial activity against Staphylococcus aureus (209), Micrococcus conglomeratus (NY), and Lactobacillus casei (ATCC 7049) of cultures acidified to pH 2.5 and sterilized for 10 minutes at 10 lbs. of steam pressure. The material having antibacterial activity was named subtilin, but was believed to consist of more than one substance. A crude concentrate was prepared by centrifuging and vacuum-drying cultures adjusted to pH 4.7. The active material was reported to be soluble in aqueous ethanol but not in 95 percent ethanol, to be diffusible, to be heat-labile under alkaline conditions but relatively stable at pH 2.5, and to be inactivated by light and by formaldehyde. (Reprints exhausted.)

Hassall, C. H., University Chemical Laboratories, Cambridge England. Subtilin C: An antibiotic concentrate from Bacillus subtilis. Nature 161 (4087): 317-318, 1948. A note confirming that B. subtilis ATCC No.

6633 produces an antibiotic active against Gram positive bacteria and mycobacteria in moderate to high dilutions. A concentrate ("subtilin C") was prepared by autoclaving dried B. subtilis cells with 80 per cent aqueous alcohol, and collecting the precipitate formed by dilution of the extract and adjustment to pH 2.3. The active factor was slowly diffusible, light-sensitive, most stable at pH 2.5 but very labile in alkaline solution, inactivated by trypsin and pepsin, and gave positive tests for α-amino acids (ninhydrin), indole nuclei (Erlich), and phenol (Folin-Denis).

Anonymous WRRL (announcements and brief abstracts of work done at the WRRL on production, purification and assay of subtilin):
Production, concentration, properties, and assay of the antibiotic, subtilin. Mimeographed Circular AIC-106, Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture. 1946. A summary of work on subtilin done at the Western Regional Research Laboratory prior to 1946.
Production of subtilin--A new antibiotic. Drug and Cosmetic Industry 60:478. 1947.
Subtilin output and purification. Oil, Paint, and Drug Reporter 151: 5, 55. 1947.
Subtilin from asparagus waste. Chem. and Engin. News 25, 1070-1071. 1947.

Lewis, J. C., Feeney, R. E., Garibaldi, J. A., Michener, H. D., Hirschmann, D. J., Traufler, D. H., Langlykke, A. F., Lightbody, H. D., Stubbs, J. J., and Humfeld, H., WRRL (authors Traufler and Langlykke, WRRL). Subtilin production in surface cultures. Arch. Biochem. 14(3): 415-425. 1947. The highest yields of subtilin in shallow-layer cultures (as determined by the antibiotic activity of aqueous ethanol extracts) were obtained with a beet molasses medium, which required the addition of $(NH_4)_2HPO_4$ (0.8 percent) and manganese (50 ppm.). [The maximum yield reported is equivalent to 770 mg. of pure subtilin per liter of culture.] Good yields were obtained on media prepared from asparagus butt waste press juice, molasses and grain worts, and corn steep liquor. An incubator temperature of 35° C., with peak temperatures in the cultures 4° to 8° C. higher, was approximately optimal. Maximum yields were usually attained in cultures 1 to 2 cm. deep after 24 to 48 hours. Prolonged incubation of asparagus and beet molasses media resulted in a drop in antibiotic activity. The distribution of subtilin between pellicle and culture filtrate varied widely for different media. Two other strains of B. subtilis did not produce appreciable amounts of subtilin. (Reprints exhausted.)

Stubbs, J. J., Feeney, R. E., Lewis, J. C., Feustel, I. C., Lightbody, H. D., and Garibaldi, J. A., WRRL. Subtilin production in submerged culture. Arch. Biochem. 14(3):427-435. 1947. Excellent yields of subtilin were produced in fermenters of one-liter capacity equipped with high-speed stirrers for dispersion of air. Asparagus-juice concentrate, when diluted to 14 percent solids, steamed for 30 minutes, adjusted to pH 7.0, inoculated and incubated at 35° C. under aeration at a rate of one volume of air per minute for 10 hours, gave the highest yield [equivalent to 700 mg. of pure subtilin per liter of culture].

Subtilin was also produced in high yield but at a slower rate on media prepared from beet molasses. (Reprints exhausted.)

Feeney, R. E., Humphreys, E. M., Lightbody, H. D., and Garibaldi, J. A., WRRL. Nutritional studies on the formation of subtilin by Bacillus subtilis in surface cultures. Fed. Amer. Socs. Expt. Biol. Proc. 6(1): 250. 1947. Preliminary announcement of results presented in the 3 following papers. (No reprints.)

Feeney, R. E., Lightbody, H. D., and Garibaldi, J. A. WRRL. Zinc as an essential element for growth and subtilin formation by Bacillus subtilis. Arch. Biochem. 15(1):13-17. 1947. Zinc was found essential for growth and subtilin formation by B. subtilis (ATCC 6633) in shallow-layer stationary cultures. A zinc-free simplified medium was prepared (by extraction with diphenylthiocarbazone in carbon tetrachloride) which permitted only slight growth and subtilin yields (less than 8 mg. of pure subtilin per liter). This medium gave a linearly increasing response in subtilin yields when 0.03 to 0.5 - 1.0 ppm. of zinc was added. Maximum yields were obtained over the range of 1 to 10 ppm. of added zinc. A partial zinc deficienty was also observed on an unpurified medium. This appears to be the first demonstration of an absolute requirement for zinc in bacterial growth. Cadmium, the only element capable of substitution for zinc. was only partially effective. (Reprints exhausted.)

Feeney, R. E., Garibaldi, J. A., and Humphreys, E. M., WRRL. Nutritional studies on subtilin formation by Bacillus subtilis. Arch. Biochem. 17(3): 435-445. 1948. The nutritional requirements for the production of relatively high levels of subtilin in shallow-layer cultures were restricted to an appropriate source of carbohydrate or related compound, inorganic sources of N, P, and S, and mineral elements. NH_3 and NO_3^- were equally satisfactory, while organic sources of N were not superior to inorganic sources. Approximate quantitative requirements were: sucrose, 5 to 7 percent; N, 2000 ppm., P, 150 ppm., and S, 80 ppm. The following simplified medium based on this study gave yields in the range of 580 to 770 mg. of subtilin (recalculated equivalent of pure subtilin) per liter of culture under submerged conditions as described previously by Stubbs et al (page 3): sucrose, 10; citric acid, 1.2; Na_2SO_4, 0.4; yeast extract, 0.5; $(NH_4)_2HPO_4$, 0.4 percent; chlorides to give K, 400; Mg, 50; Zn, 5; Fe, 5; and Mn, 5 ppm.; and NH_4OH to pH 6.8 - 6.9.

Feeney, R. E., and Garibaldi, J. A., WRRL. Studies on the mineral nutrition of the subtilin-producing strain of Bacillus subtilis. Arch. Biochem. 17(3): 447-458. 1948. The mineral requirements to produce 10 gm. of dry cells per liter of medium containing sucrose, ammonium nitrogen, asparagine, and glutamic acid were approximately as follows (in ppm.): K, 125; Mg, 2.5; Fe, 1.2; Mn, 0.7; and Zn, 0.5. The requirements for subtilin formation were generally higher than the growth requirements. Rb replaced K for growth but not for subtilin production. Except for this and a partial replacement of Zn by Cd, the requirements for the above elements were specific. Na had a detrimental effect on subtilin production, while citrate generally had a beneficial effect.

Humfeld, H., WRRL. An improved laboratory fermentor for submerged culture investigations. Jour. Bact. 54(6):689-696. 1947. A newly designed laboratory fermentor of 15 to 20 liters capacity, which gives improved air dispersion and is equipped with a mechanical foam breaker, gave a good subtilin fermentation.

Garibaldi, J. A., and Feeney, R. E., WRRL. Subtilin Production. Indus. and Engin. Chem. 41(2):432-435. 1949. Production on a 150 to 200 liter scale with asparagus juice, beet molasses, and simplified media is described. Maximum yields of 460 mg. of subtilin (recalculated equivalent of pure subtilin) per liter of medium were obtained with the sucrose-yeast extract-citrate-salts medium published previously by Feeney et al (Page 4). The fermentations were conducted at 35° C. with aeration at the rate of 1 volume of air per volume of medium per minute. Maximum antibiotic potency was obtained in 11 to 12 hours.

Purification and Chemistry

Dimick, K. P., Alderton, G., Lewis, J. C., Lightbody, H. D., and Fevold, H. L., WRRL. Purification and some properties of subtilin. Arch. Biochem. 15(1):1-11. 1947. Subtilin concentrate was obtained by extraction with 70 percent ethanol from pellicles produced by B. subtilis (ATCC 6633) on asparagus butt juice medium. The extract was concentrated in vacuo to remove ethanol, whereupon the active material precipitated. To remove inactive contaminants the dried filter cake was extracted successively with 95 percent ethanol, and with 85 percent ethanol containing 1 percent each of acetic acid and NaCl. The active material was then obtained in solution by extracting the residue with 0.16 M. acetate at pH 4.6. The extracts were treated with filtering aid (Hyflo Super-Cel)1/, deionized with exchange resins, concentrated, and lyophilized to yield a dull white powder. The subtilin preparation was soluble to at least 10 percent in salt-free acidified water, but to less than 0.5 percent at pH 6 to 9. In the presence of 0.5 percent NaCl at acid pH the solubility was less than 0.5 percent. The preparation was soluble in 0 to 80 percent ethanol, and in methanol, but not in dry ethanol, dry butanol, dry amyl alcohol, acetone, ether, petroleum ether, or chloroform. It was about 0.5 percent soluble in n-butanol saturated with water. It diffused fairly rapidly through Cellophane. The preparation contained 15.8 percent total (Kjeldahl) nitrogen. The amino nitrogen content of 1.7 percent increased to about 12.6 percent after acid hydrolysis. It contained 4.2 percent sulfur and a trace of phosphorus. It was levorotatory. Subtilin was inactivated by alkali, and the activity decreased on incubation with pepsin and trypsin. Dry subtilin preparations lost activity when stored for a period of months at room temperature, but dilute solutions at pH 2.5 appeared stable when stored in the refrigerator. The butanol extraction method described below was noted briefly. (Reprints exhausted.)

1/ The mention of this product does not imply that it is endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

Dimick, K. P., Alderton, G., Lewis, J. C., Lightbody, H. D., and Fevold, H. L., WRRL. Process for extraction and purification of subtilin. U. S. Patent 2,459,139, January 18, 1949. This patent covers the process described above by Dimick et al.

Dimick, K. P., Alderton, G., Lightbody, H. D., and Fevold, H., L., WRRL. A method for purification of subtilin. Fed. Amer. Socs. Expt. Biol. Proc. 6(1):247-248. 1947. Preliminary announcement of the method described in the following paper. (No reprints.)

Fevold, H. L., Dimick, K. P., and Klose, A. A., WRRL. Isolation of subtilin from submerged cultures. Arch. Biochem. 18(1):27-34, 1949. A method for the isolation of subtilin is described which depends on the extraction of the culture broth at pH 2 with one-half volume of n-butanol, and separation of the two phases in a Sharples centrifuge. Subtilin is recovered from the butanol phase (a) by addition of one-half volume of petroleum ether to the butanol extract and extraction with 1 percent acetic acid followed by precipitation of subtilin from the aqueous extract with 10 percent NaCl, or (b) by addition of NaCl as described below by Lineweaver et al followed by recovery of the precipitate in the Sharples centrifuge. The moist cake of crude subtilin is extracted twice with 10 volumes of absolute ethanol. Toxic impurities remaining are removed by dissolving to give a 0.5 to 0.75 percent solution of the extracted cake at pH 3.0 to 3.5, adding NaCl to 0.4 percent, and adjusting to pH 4.6 with 0.1 percent NaOH. The flocculant precipitate is removed and re-extracted twice in the same fashion. Subtilin is recovered from the combined extracts by precipitation with 10 percent NaCl, removal of excess NaCl by washing with 1 percent NaCl and then with absolute ethanol, and drying. About 50 percent over-all yields were obtained. The product appeared to be essentially homogeneous by fractional dialysis, salt fractionation, and electrophoretic studies. The LD_{50} for subcutaneous injection in mice was about 3 gm. per kg. of body weight. [The maximum potency obtained was about 90 percent of that of pure subtilin.]

Dimick, K. P., Stubbs, J. J., Garibaldi, J. A., Lightbody, H. D., and Fevold, H. L., WRRL. Purification of subtilin. U. S. Patent 2,476,085, July 12, 1949. This patent covers the process described above by Fevold, et al.

Lineweaver, H., Klose, A. A., and Alderton, G., WRRL. An alternate step for the isolation of subtilin. Arch. Biochem. 16(2):311-313. 1948. Subtilin may be salted out of butanol extracts of B. subtilis (ATCC 6633) culture by adding 60 gm. of NaCl per liter of butanol and adjusting the pH of the aqueous phase to 5. The precipitate may be collected in the bowl of a Sharples continuous centrifuge which may be adjusted to remove separately the dehydrated butanol and the salt-water. Relatively little subtilin is lost in these liquid phases.

Lineweaver, H., Klose, A. A., and Alderton, G., WRRL. Technique for isolating subtilin. U. S. Patent 2,481,763, September 13, 1949. This patent covers the process described above by Lineweaver et al.

Lewis, J. C., and Jansen, E. F., WRRL. Enhancement of subtilin activity by methylation. Fed. Amer. Socs. Expt. Biol. Proc. 6(1):270, 1947. Preliminary announcement of work described in the following paper. (no reprints.).

Carson, J. F., Jansen, E. F., and Lewis, J. C., WRRL. Esterification of subtilin and its effect on solubility and in vitro bacteriostatic activity. Jour. Amer. Chem. Soc. 71(7):2318-2322. 1949. The preparation of methyl, ethyl, hydroxyethyl, and hydroxypropyl esters of subtilin is described. Esterification with methanol in the presence of 0.03 N HCl at 0° C. is specific for carboxyl groups, a maximum of 6 to 7 equivalents of ester being introduced per 10^4 gm. of subtilin. These esters have their solubility in 0.9 percent NaCl at pH 7.3 increased approximately two-fold and their bacteriostatic activity for Streptococcus faecalis (ATCC 7080) and Micrococcus conglomeratus (MY) increased 2- to 4-fold. If the esterification is carried out at 25° C. amide nitrogen is decreased and as much as 14 equivalents of ester may be introduced per 10^4 gm. of subtilin. With 9 to 11 equivalents the solubility in 0.9 percent NaCl at pH 7.3 increases 30- to 50-fold to 3 to 5 percent. Certain hydroxyethyl esters were characterized by increased bacteriostatic activity or by greatly increased solubility. Ethyl and hydroxypropyl esters did not show increased bacteriostatic activity, and were characterized by only slight or moderate increases in solubility in physiological salt solution.

Lewis, J. C., and Jansen, E. F., WRRL. Alcohol derivatives of subtilin. U. S. Patent 2,495,743, January 31, 1950. This patent covers the subtilin esters containing up to 7 equivalents of methoxyl groups per 10^4 gm. of subtilin.

Lewis, J. C., and Alderton, G., WRRL. Amino acid composition of subtilin. (Paper presented at the September, 1948 meeting of the American Chemical Society.) Microbiological and chemical assays of subtilin for the amino acids found generally in proteins led to the following incomplete empirical formula: glycine (4), alanine (2), L-valine (2), L-isoleucine (2), L-leucine (8), L-phenylalanine (2), L-proline (2), tryptophane (2), L-lysine (5), aspartic acid (2), L-glutamic acid (5), N (73), $-NH_2$ (van Slyke) (8), $-CONH_2$ (10), S (10), -COOH (4). The numbers in parentheses indicate the number of gram residues or gram atoms in 6500 grams of subtilin containing 15.8 percent nitrogen and 4.8 percent sulfur. 82 percent of the total nitrogen has been accounted for as generally occurring amino acids and as amide nitrogen. Cystine, methionine, serine, threonine, hydroxyproline, arginine, histidine, tyrosine, D-leucine, D-glutamic acid, P, -SH, -SS-, $-SCH_3$, $-OCH_3$, and $-OC_2H_5$ are absent. Two S-containing moieties have been isolated from 6 N hydrochloric acid hydrolyzates of subtilin. One, obtained in yield equivalent to 10 percent of the total sulfur, has been identified as lanthionine. The other, obtained in similar yield, has the empirical formula $C_7H_{14}O_4N_2S$. Work on identification of the S-containing moieties is being continued. (No reprints.)

Stansly, P. G., and Ananenko, N. H., American Cyanamid Co, Stamford, Conn. Resistance of polymixin to some proteolytic enzymes. Arch.

Biochem. 15(3): 473-474. 1947. Unlike polymyxin, subtilin was largely inactivated by pepsin (pH 2.2 - 4.6), trypsin (pH 4.4 - 7.5), pancreatin (pH 4.4 - 7.5), and erepsin (pH 6.1 - 7.8).

Biological Activity

Salle, A. J., and Jann, G. J., University of California at Los Angeles. Subtilin--An antibiotic produced by Bacillus subtilis. I. Action on various organisms. Proc. Soc. Expt. Biol. and Med. 60:60-64. 1945. Partially purified subtilin was found to inhibit growth of a number of Gram-positive bacteria, the Gram-negative Neisseria catarrhalis and N. gonorrhoeae, certain acid fast bacteria including Mycobacterium tuberculosis, and certain pathogenic fungi. The effect on M. tuberculosis was bacteriostatic at low and bactericidal at higher concentrations.

Salle, A. J., and Jann, G. J., Univeristy of California at Los Angeles. Subtilin--Antibiotic produced by Bacillus subtilis. II. Toxicity of subtilin to living embryonic tissue. Proc. Soc. Expt. Biol. and Med. 61: 23-24. 1946. Partially purified subtilin possessed a low toxicity for embryonic chick heart tissue cultures. Exposure to a 0.2 percent solution for 10 minutes at 37° C. was required to kill the cultures. A concentration of 0.01 percent was required to kill Staphylococcus aureus under similar conditions.

Salle, A. J., and Jann, G. J., University of California at Los Angeles. Subtilin--Antibiotic produced by Bacillus subtilis. III. Effect on type III pneumococcus in mice. Proc. Soc. Expt. Biol. and Med. 62: 40-42. 1946. Partially purified subtilin in repeated doses of 0.05 mg. injected intraperitoneally quickly cured experimental type III pneumococcus infections induced in mice by intraperitoneal inoculation, without apparent toxic reaction in the animals. A group untreated until 9 hours after infection were in very bad condition before treatment, but appeared almost normal after 2 or 3 injections.

Salle, A. J., and Jann, G. J., University of California at Los Angeles. Subtilin--Antibiotic produced by Bacillus subtilis. IV. Effect of subtilin on the course of experimental anthrax infections in guinea pigs. Proc. Soc. Expt. Biol. and Med. 63: 41-42. 1946. Partially purified subtilin in doses of 6 mg. injected intraperitoneally at frequent intervals over a period of 11 days protected guinea pigs from experimental anthrax infections induced by intraperitoneal inoculation, without apparent toxic reaction in the animals. Initial treatments made 3, 6, and 9 hours after inoculation. Untreated controls died in about 3 days.

Salle, A. J., and Jann, G. J., University of California at Los Angeles. Subtilin--Antibiotic produced by Bacillus subtilis. V. Effect on Streptococcus pyogenes infections in mice. Proc. Soc. Expt. Biol. and Med. 63: 519-520. 1946. Partially purified subtilinnin 0.1 or 0.2 mg. repeated doses injected intraperitoneally quickly cured experimental Streptococcus pyogenes infections induced in mice by intraperitoneal inoculation, without apparent toxic reaction in the animals.

Animals first given subtilin 9 hours after inoculation were in an advanced stage of disease but recovered promptly.

Salle, A. J., University of California at Los Angeles. The nature, properties and toxicity of subtilin, and its chemotherapeutic effect on the course of experimental infections in animals. Presentation at the Conference on Antibiotic Research, Washington, D. C., Jan. 31-Feb. 1, 1947, under the auspices of the Antibiotics Study Section of the National Institute of Health. A review of AIC-106 (see p. 3) and of published (see Salle and Jann, above) and unpublished original work. Partially purified subtilin in twice repeated 1 mg. doses injected intraperitoneally quickly cured intraperitoneally induced Staphylococcus aureus infections in mice. Definite suppressive action on experimental tuberculosis infections in guinea pigs was claimed (see below).

Salle, A. J., and Jann, G. J., University of California at Los Angeles. Preparation of a modified subtilin suitable for the treatment of tuberculosis and other infections in animals. Jour. Clin. Invest. 28(5): 1036-1038. 1949. A solution of partially purified subtilin in 10 percent urea, from which part of the impurities are believed to be removed by addition of pectin, is stated not to produce nodule-like deposits when injected subcutaneously in guinea pigs. Tuberculosis-infected guinea pigs treated for 6 weeks with a total of 22 mg. of subtilin per day (in three subcutaneous injections) showed relatively few lesions in liver, lungs, and spleen when one lot of subtilin was used, but relatively little tuberculostatic effect was noted when another lot of subtilin was used. Animals treated simultaneously with subtilin and with a sub-optimal dosage of streptomycin showed much less tuberculous involvement than with either substance alone.

Salle, A. J., and Jann, G. J., University of California at Los Angeles. Studies on subtilin fastness in vitro. Jour. Bact. 55(4): 463-469. 1948. Staphylococcus aureus became resistant to 400 times the concentration of subtilin initially bacteriostatic (2.5 ppm. of partially purified subtilin) after 6 serial transfers in liquid medium containing increasing concentrations of subtilin. Similarly Mycobacterium phlei, initially sensitive to 2.5 ppm., became resistant to 500 ppm. after 9 transfers, and Escherichia communior, initially sensitive to 300 ppm., became resistant to 1000 ppm. after 3 transfers. The resistant strains tended to retain much of their resistance for as many as 75 daily transfers in liquid medium free of subtilin.

Anderson, H. H., Villela, G. G., Hansen, E. L., and Reed, R. K., University of California Medical School, San Francisco. Some physical and biologic properties of subtilin and other antibiotics. Science 103(2675): 419-420. 1946. Partially purified subtilin proved active in vitro against Lactobacillus plantarum, Endamoeba histolytica and its associated bacterium 't', and Trypansoma equiperdum, but not against Ascaris suis or Leishmania donovani (also tested in vivo). The concentrations required for antibiotic effect also lowered surface tension appreciably. Intravenous injection of 1 percent solution in mice gave an LD_{50} of 60 ± 3 mg. per kg.; subcutaneous injection gave

LD_{50} pf 670 ≠ 30 mg. per kg. When given intragastrically, 5.0 grams/kg. killed. One percent solution instilled into the rabbit's eye was nonirritating.

Chin, Y. C., University of California Medical School, San Francisco. Biologic properties of subtilin in physiologic saline solution. Fed. Amer. Socs. Expt. Biol. Proc. 6(1):317. 1947. The toxicity of partially purified subtilin could be reduced without loss of bacteriostatic or amebacidal potency by extracting with 0.9 percent sodium chloride solution. The extract contained the antimicrobial activity.

Anderson, H. H., and Wong, S. C., University of California Medical School, San Francisco. Antibiotics in experimental tuberculosis. Tuberculology 8 (3): 77-82. 1946. Partially purified subtilin at 2.5 ppm. prevented growth of virulent strains of Mycobacterium tuberculosis in a modified Dubos medium. The partially purified preparations used gave a marked anaphylactic reaction with guinea pigs. Topical application of subtilin was ineffective in treating experimentally induced tuberculous infections of the cornea of rabbits. Daily subcutaneous injections of 6 mg. of subtilin continued over a period of 6 weeks failed to affect the course of experimentally induced tuberculosis in Syrian hamsters. Therapy was initiated 8 days after infection. One particular lot of subtilin was well tolerated; two others gave marked local tissue toxicity. It was believed that failure of therapy was related to precipitation by physiological concentrations of NaCl. Such concentrations reduced the in vitro antibiotic activity of subtilin on Lactobacillus planatarum.

Wong, S. C., Hambly, A. S., and Anderson, H. H., University of California Medical School, San Francisco. Use of modified Dubos and Davis medium for demonstration of antibiotic activity of subtilin against Mycobacterium tuberculosis. Jour. Lab. Clin. Med. 32(7): 837-841. 1947. In Dubos medium partially purified subtilin was bacteriostatic at 1/400,000 dilution, and bactericidal only at 1/20,000 or lower dilution. A virulent strain of M. tuberculosis was used.

Anderson, H. H., and Chin, Y. C., University of California Medical School, San Francisco. Antibiotic activity of subtilin and streptomycin in the presence of BAL. Science 106 (2765): 643-644. 1947. BAL (2,3-dimercaptopropanol) at a dilution of 1/100,000 in Dubos medium increased the bacteriostatic potency of subtilin against 3 pathogenic and 1 non-pathogenic strains of Mycobacterium tuberculosis by 2 to 16 times, and of streptomycin to a lesser degree.

Anderson, H. H., University of California Medical School, San Francisco. New surface active antibiotics. Jour. Invest. Dermatology 8(1): 25-33. 1947. Certain wetting agents, "Tergitol" and "Tween 80", substantially increased the bacteriostatic action of subtilin against Lactobacillus plantarum. Sodium lauryl sulfate, on the other hand, greatly reduced the activity of subtilin. Serum albumin (5 percent) or globulin (1 percent) did not repress the activity of subtilin. In Dubos medium with 0.05 percent of Tween 80, partially purified subtilin inhibited growth of Mycobacterium tuberculosis for 12 days at

1/400,000 dilution. Subtilin ruptures the cell membrane of Endamoeba histolytica, in vitro.

Chin, Y. C., University of California Medical School, San Francisco. Effect of some derivatives of subtilin on tubercle bacilli and rabbit leukocytes in vitro. Fed. Amer. Socs. Expt. Biol. Proc. 7(1): 211-212. 1948. The tuberculostatic activity of methylated, ethylated and glycolated derivatives of subtilin against Mycobacterium tuberculosis ($H37R_V$) in Dubos medium was either no different from subtilin or was increased 2-fold. The low toxicity toward rabbit leukocytes was unchanged, except for a 3-fold increase for one sample.

Farber, S. M., Eagle, H. R., Anderson, H. H., and Gorman, R. D., University of California Medical School, San Francisco. Topical application of subtilin to tuberculous lesions in man. Jour. Lab. Clin. Med. 33(7): 799-804. 1948. Subtilin solutions were administered by inhalation to 8 tuberculous patients. Amounts ranging from 4 to 50 gms. were used for periods of 2.5 to 10 months. Suggestive evidence of therapeutic activity was observed in 3 cases. No evidence of s serious toxicity was observed.

Steenkin, W., and Wolinsky, E., Trudeau Laboratory, Trudeau, New York. The tuberculostatic effect of subtilin in vitro and in vivo. Jour. Bact. 57(4): 453-457. 1949. Partially purified subtilin inhibited the $H37R_V$ strain of tubercle bacillus when used in moderate concentrations (10 to 100 ppm.) in medium containing sorbitan monooleate, but not in other media lacking sorbitan monooleate which gave granular growth of the bacillus. Tuberculous guinea pigs treated intramuscularly with 1 to 2 mg. of subtilin every 12 hrs. for 64 days exhibited as much gross tuberculous disease on autopsy as did the controls. Four mg. of this lot of partially purified subtilin per animal of 400 to 600 gm. per day was near the maximum tolerated dose, since progressive weight losses were observed.

Dubos, R. J., Rockefeller Institute. The effect of wetting agents on the growth and susceptibility of tubercle bacilli. Proc. 48th General Meeting Society of American Bacteriologists, May 1948, pp. 83-84. Certain arylalkyl polyethers of phenols wet the surfaces of tubercle bacilli without affecting their metabolism as do the water-dispersible esters of oleic, stearic, and palmitic acids (Tweens) which are more readily hydrolyzed by tissue enzymes. All surface-active substances tested which promote dispersed growth also increase susceptibility to a wide variety of anitbacterial agents. For example, strains of mycobacteria which grow in oleic acid albumin medium in the presence of 100 ppm. of subtilin are inhibited by 5 ppm. in media containing the proper wetting agent.

Knight, V., and Tompsett, R., Cornell University Medical College. The relation of growth dispersion to growth inhibition of M. tuberculosis by subtilin and other chemotherapeutic agents. Jour. Clin. Invest. 27: 544. 1948. Knight, V., Schultz, S., and Dubois, R. The relation of type of growth of Mycobacterium tuberculosis to the

antituberculous activity of subtilin. Proc. 48th General Meeting Society of American Bacteriologists, May 1948, p. 84. Subtilin is highly inhibitory to pneumococci, streptococci, and tubercle bacilli in vitro, and has a high therapeutic activity for infections of the first two organisms but not for tuberculosis in mice. It was found that subtilin inhibits the tubercle bacillus only when dispersed growth is obtained (by addition of non-ionic wetting agents (Tween)). The inhibitive concentration of subtilin in the presence of Tween is 3 to 12 ppm. Serum antagonized the action of the wetting agent and permits clumped growth. Under this condition subtilin does not inhibit growth at 100 ppm. If sufficient wetting agent is used antitubercular activity may be demonstrated in serum from subtilin-treated rabbits.

Eagle, H., Musselman, A. D., and Fleischman, R., United States Public Health Service. The action of bacitracin and subtilin on Treponema pallidum in vitro and in vivo. Jour. Bact. 55(3): 347-358. 1948. Subtilin proved about one-half as active as penicillin G against Streptoccus pyogenes (C-203), 0.03 ppm. inhibiting in vitro. Against T. pallidum (Reiter strain) subtilin was approximately 0.01 as active in vitro as penicillin G, 2 to 4 ppm. being necessary for bacteriocidal action. Subtilin at 10 mg. per kg. administered intramuscularly 4 times daily for 4 days failed to eliminate the treponemata from the testicular chancres in rabbits.

Kraft, M. E., and Spencer, G. R., University of Wisconsin. In vitro effect of certain antibacterial agents on organisms encountered in bovine mastitis. Proc. Soc. Expt. Biol. Med. 70: 176-179. 1949. Subtilin [80 percent pure] killed 3 Gram-positive bacteria (S. aureus, Str. agalactiae, and C. pyogenes) associated with bovine mastitis at a dilution of 1:80,000, and inhibited growth at twice this dilution. Gram-negative bacteria were not affected.

Wilson, R. H., Lewis, J. C., and Humphreys, E. M., Pharmacology Laboratory and WRRL. Subtilin in blood after parenteral administration. Fed. Amer. Socs. Expt. Biol. Proc. 7(1): 266. 1948. Preliminary announcement of results presented in the following paper.

Wilson, R. H., Humphreys, E. M., Reynolds, D. M., and Lewis, J. C., Pharmacology Laboratory and WRRL. Absorption of subtilin in the rabbit. Proc. Soc. Expt. Biol. Med. 71: 700-705. 1949. Subtilin [80 to 90 percent pure] had a very low toxicity when administered subcutaneously; 3 gm. per kg. of body weight did not kill mice. Because of its low solubility in physiological saline or serum, subtilin administered subcutaneously or intramuscularly precipitates at the site of injection; blood levels ranging from a trace to 1.4 ppm. were obtained from a dose of 100 mg. per kg. A sterile abcess was still present in muscles 2 months after subtilin injection. Intraperitoneal and oral administration gave no higher levels. Two to 4 ppm. was found in the blood after rectal administration. Intravenous administration gave blood levels of 100 to 200 ppm. of subtilin, which fell to 10 to 30 ppm. in 2 hrs. and to zero in 24 hrs. As much as 7 percent of the subtilin was found in the urine collected within 2 hours. High levels were

maintained for 4 hrs. by intravenous infusion without apparent harm. Subtilin methyl esters (5.9 and 11.9) equivalents of OCH_3 per 10^4 gm.) gave no higher blood levels than did subtilin. Sensitive bacteria for the cup-plate assay of subtilin are mentioned (20 mm. zones with 1 to 2 ppm.).

Andersen, A. A., and Michener, H. D., WRRL. Preservation of foods with antibiotics. I. Preservation of vegetables with subtilin and mild heat treatment. Food Technology (submitted). The combination of 5 to 10 ppm. of subtilin [80 to 90 percent pure] and mild heat treatment (100° C. for a few minutes) served to protect canned vegetables against microbial spoilage as well as the sterilization under steam pressure required by conventional practice. Peas, asparagus, corn, green beans, peeled potatoes, tomato juice, and milk have been processed successfully by this technique. In some cases the vegetables were inoculated with Bacillus stearothermophilus, a thermophile which frequently causes loss in commercial practice.

Goodman, J. J., and Henry, A. W., University of Alberta, Edmonton, Canada. Action of subtilin in reducing infection by a seed-borne pathogen. Science 105: 320-321. 1947. Barley seeds exposed simultaneously to a 0.1 percent solution of partially purified subtilin and Xanthomonas translucens cerealis for 24 hours did not yield infected seedlings, but with 0.02 percent subtilin infection approached that of controls without subtilin. Only partial protection was obtained when the seeds were infected, dried, and subsequently treated with subtilin.

Related Compounds

Michener, H. D., and Snell, N., WRRL. Two antifungal substances from Bacillus subtilis cultures. Arch. Biochem. 22(2): 208-214. 1949. Cultures of B. subtilis ATCC #6633 possessed antifungal activity which was not exhibitedbby partially purified subtilin preparations. A single extraction with butanol at pH 2.5 removed most of the activity against Aspergillus niger but not against Rhizopus solani, thus indicating that at least two antifungal substances were present. Concentration procedures are given.

Walton, R. B., and Woodruff, H. B., Merck & Co., Rahway, New Jersey. A crystalline antifungal agent, mycosubtilin, isolated from subtilin broth. Jour. Clin. Invest. 28(5): 924-926. 1949. B. subtilis ATCC #6633 broth yielded a white crystal of "mycosubtilin", melting point 256-257° C., insoluble in all reagents tested except in pyridine, warm 70 percent ethanol, and dilute NaOH. The acid hydrolyzate when assayed microbiologically yeilded 45 percent of aspartic acid, 5.4 percent of tyrosine, and 4.2 percent of proline. Mycosubtilin is heat stable. 1.5 ppm. inhibits the growth of a Trichophyton strain (MF301) on agar, although 200 ppm. of subtilin is inactive. The action is neutralized by the addition of 10 percent of horse serum. Mycosubtilin inhibits the growth of many but not all filamentous fungi and yeasts at 1.5 to 20 ppm.

Supplement
(added Oct., 1950)

Macmorine, H. G., and Slinn, G. S., Connaught Medical Research Laboratories, Univ. of Toronto. Plate techniques for the assay of subtilin. Canad. J. Public Health 39:203-208. 1948. Diffusion plate assays which utilize Staphylococcus aureus 209P are described. Size of zones was increased by allowing a diffusion period before incubation during which the plates were refrigerated, and by incorporating 0.1 percent of Tween 80 in the seed layer. Filter paper discs were preferred to cups. The plate tests were only 1 to 2 percent as sensitive as turbidimetric tests, but were more reproducible.

Feeney, R. E., and Garibaldi, J. A., WRRL. Method of preparing subtilin. U.S. Patent 2,508,378, May 23, 1950. Covers the conditions of mineral nutrition described by Feeney et al. (1949) cited on page 4 of this bibliography.

Stubbs, J. J., Feeney, R. E., Garibaldi, J. A., and Feustel, I. C., WRRL. Process of producing subtilin. U.S. Patent to be granted Oct. 1950. Covers the production of subtilin under submerged conditions as described by Stubbs et al. (1947), cited on page 3 of this bibliography.

Brink, N. G., Mayfield, J., and Folkers, K. Research Laboratories of Merck & Co., Rahway, N.J. The purification of subtilin concentrates by counter-current distribution. In manuscript, 1950. Subtilin of the highest potency isolated as described by Fevold et al. (1949) (see page 6 of this bibliography) was distributed into 12 to 24 fractions between n-butanol and water or sec-butanol and water. A small amount of material of low bacteriostatic potency was separated, while the potency of the most active fractions was increased by approximately 10 percent over that of the starting material. Some physical and chemical constants for the most active fractions are reported.

Carson, J. F., and Lewis, J. C., WRRL. Methyl esters of subtilin. U.S. Patent 2,520,908, Sept. 5, 1950. Covers the highly soluble subtilin esters containing more than 7 equivalents of methoxyl groups per 10^4 gm. of subtilin as described by Carson et al. (1949), cited on page 7 of this bibliography.

Alderton, G., and Fevold, H. L., WRRL. Lanthionine in subtilin. Jour. Amer. Chem. Soc., in press. Meso-lanthionine was isolated from hydrochloric acid hydrolyzates of subtilin in yield equivalent to approximately 10 percent of the sulfur. It was found identical with meso-lanthionine isolated from alkali-treated hair, by X-ray powder photographs, by degradation of the benzoyl derivative with Raney nickel, and through the benzoyl and carbobenzoxy derivatives.

D'Arcy Hart, P., and Moss, B., National Institute for Medical Research, Mill Hill, London, N.W. 7. The distinction of licheniformin from subtilin by cross-reactions with antibiotic-resistant strains of Mycobacterium phlei. Jour. Gen. Microb. 4:244-256. 1950. Licheniformin and subtilin induced resistance in Mycobacterium phlei more slowly and to a much smaller degree than did streptomycin. Strains resistant to licheniformin and subtilin tended to revert, whereas streptomycin-resistance was stable. Licheniformin resistance was somewhat more difficult to induce and less stable than subtilin resistance. Cross-resistance tests of bacteriostatic activity between the resistant strains and the three antibiotics distinguished completely licheniformin from both subtilin and streptomycin. Although the difference between licheniformin and subtilin was largely confirmed by a more sensitive bactericidal test, with viable counts after varying periods of exposure of the resistant strains to the antibiotics in nutrient medium, the test, nevertheless, revealed a minor overlap between the two antibiotics, which was either the result of contamination of one by the other or, more probably, the expression of some common chemical property. Licheniformin and subtilin differed in the speed of their lethal action on M. phlei, subtilin being much the slower.

Knight, V., and Tompsett, R., Cornell University Medical College. Relationship of type of growth of M. tuberculosis to antituberculous activity of subtilin. Proc. Soc. Exp. Biol. Med. 73:55-60. 1950. Detailed presentation of the results summarized in the abstracts cited on pp. 11-12 of this bibliography.

Heishman, J. O., Bur. of Animal Industry, U.S.D.A., Beltsville, Md. Report on subtilin and bacitracin as possible treatment for bovine mastitis. Amer. Jour. Vet. Res. 11:206-210. 1950. Although subtilin was active in vitro against micro-organisms associated with bovine mastitis, subtilin in amounts up to 0.4 gm. per quarter in two doses caused elimination of the infection from only two of 15 quarters infected with Streptococcus agalactiae, and had no permanent effect on the infection with Str. disgalactiae (3 quarters) or with hemolytic Staphylococcus (4 quarters).